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Biochimica et Biophysica Acta 1455 (1999) 54–60

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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β-Trace protein in human cerebrospinal fluid: a diagnostic marker for *N*-glycosylation defects in brain

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Received 29 March 1999; received in revised form 1 July 1999; accepted 2 July 1999

Abstract

As carbohydrate-deficient glycoprotein syndromes (CDGS) are multisystemic disorders with impaired central nervous function in nearly all cases, we tested isoforms of β-trace protein (βTP), a 'brain-type' glycosylated protein in cerebrospinal fluid (CSF) of nine patients with the characteristic CDGS type I pattern of serum transferrin. Whereas the serum transferrin pattern did not discriminate between the various subtypes of CDGS type I (CDGS type Ia, type Ic and patients with unknown defect), βTP isoforms of CDGS type Ia patients differed from that of the other CDGS type I patients. The percentage of abnormal βTP isoforms correlated with the severity of the neurological symptoms. Furthermore, two patients are described, who illustrate that abnormal protein *N*-glycosylation can occur restricted to either the 'peripheral' serum or the central nervous system compartment. This is the first report presenting evidence for an *N*-glycosylation defect restricted to the brain. Testing βTP isoforms is a useful tool to detect protein *N*-glycosylation disorders in the central nervous system.

Keywords: Human β-trace protein; 'Brain-type' glycosylation; Carbohydrate deficient glycoprotein syndrome; CDGS type I; Phosphomannomutase deficiency

1. Introduction

The carbohydrate-deficient glycoprotein syndromes (CDGS) are a new group of inherited disorders affecting the synthesis of the asparagine-linked (*N*-linked) glycans first described by Jaeken in 1980 [1]. Based on a different banding pattern in the isoelectric focusing (IEF) of serum transferrin due to the abnormal glycosylation of the oligosaccharide chains, four types of CDGS (type I–IV) have been classified [2–6]. CDGS type I syndromes are defined by a type I sialotransferrin pattern delineated by increased di- and asialotransferrin and decreased tetra-

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0925-4439/99/$ – see front matter
PII: S0925-4439(99)00078-2

sialotransferrin bands. Among CDGS type I three different diseases have been identified: CDGS type Ia is caused by phosphomannomutase 2 (PMM 2) deficiency [7] and CDGS type Ib by phosphomannose isomerase (PMI) deficiency [8–10]. Both cytosolic enzymes catalyse steps in the early glycosylation pathway. In a group of four patients (children of an inbred family) and another unrelated child, a defective glycosyltransferase of the endoplasmic reticulum (ER) causes the accumulation of dolichol-linked $Man_9GlcNAc_2$. As for historical reasons the classification of CDGS is based on the isoform pattern observed for transferrin, the disorder is called CDGS type Ic in this publication and in two earlier papers [11,12], whereas Körner et al. published their patient with the same enzyme defect as CDGS type V [13]. In addition, a number of patients with a CDGS type I transferrin pattern on IEF but so far unknown *N*-glycosylation defect have been identified. They are labelled CDGS type Ix.

Beta-trace protein (βTP) is one of the most prominent polypeptide constituents in human cerebrospinal fluid (CSF), detectable in a concentration of about 8 μg/ml [14]. This glycoprotein is predominantly intrathecally synthesised and secreted by glia-rich fractions of the brain. It is also detectable in trace amounts in kidney, epididymis and other organs [15,16]. The structure of the carbohydrate units of βTP was elucidated only recently. There is evidence that βTP is 'brain-type' glycosylated. This means that all biantennary *N*-linked oligosaccharides are of the complex type and have a high degree of peripheral fucosylation, high amounts of bisecting *N*-acetylglucosamine (GlcNAc) and *N*-acetylneuraminic acid (NeuNAc) in α2,3 or α2,6 linkage [17]. In contrast to these characteristics of βTP in CSF, peripheral fucosylation and bisecting GlcNAc are nearly absent in serum-type glycoproteins and NeuNAc is found predominantly in α2,6 linkage [18,19].

CDGS are multisystemic diseases and the common clinical presentation in the most frequently occurring CDGS type Ia, but also in CDGS types Ic and Ix, is moderate to severe cerebral symptoms. Under the assumption that abnormal glycosylation in brain may play a role in the pathogenesis of brain dysfunction in CDGS, the question was whether in certain CDG syndromes the abnormal glycosylation pattern of serum glycoproteins can be recovered also in 'brain-type' glycosylated glycoproteins such as βTP. Furthermore, based on the idea that deficient glycosylation as a cause of brain damage might be confined to the CNS [20], we tested βTP for abnormal glycosylation in 500 CSF samples of children with impaired cerebral function of unknown cause.

2. Materials and methods

2.1. Materials

2.1.1. Controls

In 25 age-matched controls CSF was obtained as part of routine diagnostic procedure. Retrospectively, the controls were classified as suitable reference subjects because of lack of evidence for immunological or renal diseases. Appropriate tests ruled out acute or chronic infectious diseases. Serum and CSF samples were kept at −80°C until analysis.

2.1.2. CDGS type Ia, Ic and Ix

Samples of CSF and serum were obtained from children with CDGS after their parents' informed consent. In nine CDGS type I patients (4 type Ia, 1 type Ic and 4 type Ix cases), aged between 3 months and 7 years, the serum transferrin pattern on IEF showed the characteristic increase of a- and disialotransferrin and decrease of tetrasialotransferrin (Table 1). The characteristic abnormalities on IEF were also demonstrated in the IEF pattern of TBG (thyroxine binding globulin) confirming a generalised glycoprotein abnormality (data not shown). A variant in the protein moiety of transferrin was excluded by IEF of serum transferrin after neuraminidase treatment [21]. Four CDGS type Ia patients showed reduced PMM activity in leukocytes and fibroblasts (leukocytes 0.0–0.1 mU/mg protein, fibroblasts 0.09–0.57 mU/mg protein, normal ranges: leukocytes 0.41–1.81 mU/mg protein, fibroblasts 1.27–4.53 mU/mg protein). By SSCP analysis and sequencing the frequent missense mutation F119L/R141H was identified in two patients, and R123Q/R162W was found in another child [22]. In the fourth subject a new missense mutation H218L was described in combination with a splice mutation in intron 3. In CDGS

Table 1
Relative distribution (%) of serum sialotransferrin fractions on isoelectric focusing of different CDGS type I patients, patients H.K. and C.V., and healthy controls

Sialotransferrin fraction	Controls (range) ($n=30$)	CDGS type Ia ($n=4$)	CDGS type Ic ($n=1$)	CDGS type Ix ($n=4$)	H.K.	C.V.
0	0.0–2.6	6.6–29.5	10.7	2.2–16.8	2.2	3.6
1	0.0–2.6	1.1–5.4	2.0	1.4–3.3	4.8	2.3
2	1.6–6.1	23.1–37.7	34.9	20.7–42.7	19.2	8.3
3	2.5–15.6	5.9–9.6	6.1	8.0–13.3	28.4	18.2
4	51.2–72.2	20.6–42.3	33.9	22.5–49.0	35.6	48.1
5	12.1–30.8	6.0–14.8	11.0	6.7–14.7	9.8	16.5
6	0.0–9.0	0.0–2.8	1.6	0.0	0.0	2.8

type Ic and CDGS type Ix cases, PMM and PMI deficiency was excluded by measuring the enzyme activities in leukocytes and fibroblasts. Biochemical and molecular genetic characterisation of the defect in the glycosylation of dolichol-linked oligosaccharides in the CDGS type Ic family has been described [11,12]. The basic defect(s) in the CDGS type Ix cases remain(s) unclear and may turn out to be heterogeneous.

In all patients with CDGS type I the CNS was involved. The neurological abnormalities were highly variable, ranging from only minor neurological symptoms to full-blown neuropathology of CDGS type Ia. Muscular hypotonia, psychomotor retardation and speech delay were constant features. Overall, CDGS type Ia patients presented with more severe neurological symptoms compared to patients with CDGS type Ic and Ix.

2.1.3. CSF from a patient with other hypoglycosylation of serum glycoproteins

During the workup of a 21-year-old female (H.K.) with severe mental retardation and neurological impairment, an abnormal pattern of the serum transferrin and TBG was obtained on IEF. Also in this patient, a polymorphism in the transferrin could be ruled out as an explanation for the abnormal isoforms distribution after neuraminidase incubation. The sialotransferrin pattern showed elevated mono-, di- and trisialo- and decreased tetrasialotransferrin bands, by definition a CDGS type II pattern (Table 1). This patient presented with an Angelman-like appearance, severe developmental and speech delay and mild epilepsy. Magnetic resonance imaging of the brain showed no abnormalities.

2.1.4. CSF from children with various neurological disorders

A total of 500 CSF samples were collected from children (age 4 months–18 years) with various neurological symptoms. This material was obtained in the context of diagnostic investigations and was stored until analysis at −80°C. The leading neurological signs and symptoms included mental and motor retardation, epilepsy and muscular hypotonia.

2.2. Methods

2.2.1. Isoelectric focusing of serum transferrin

IEF of serum transferrin was carried out essentially as described by van Eijk and van Noort [23]. Iron-saturated serum was separated on a hydrated immobiline gel (pH 4–7) on a Ultrophore system (Pharmacia, Uppsala, Sweden). After IEF the transferrin isoforms were visualised by adding rabbit anti human transferrin antibodies (Dako, Glostrup, Denmark) and the gels were stained with Coomassie blue. The relative amounts of transferrin isoforms were quantified with the Ultrascan XL laser densitometer.

2.2.2. IEF of thyroxine-binding globulin in serum

Pure serum was added on a PhastGel (pH 4–6.5, Pharmacia Biotech) and separated on a PhastSystem. After addition of rabbit anti-human TBG antibody (Dako) the TBG pattern was determined by silver staining.

2.2.3. Phosphomannomutase (PMM)- and phosphomannose isomerase (PMI)-assay

PMM and PMI activities were measured in leuko-

cytes and/or fibroblasts, according to the procedure described by Van Schaftingen and Jaeken [7].

2.2.4. SDS–PAGE and immunoblotting of β-trace protein

After ethanol precipitation of protein from 100 μl CSF and centrifugation, the pellets were dissolved in 20 μl SDS-buffer, containing 10.0 mM Tris–HCl, 1.0 mM EDTA, 2.5% SDS, 5% β-mercaptoethanol and 0.01% Bromophenol blue, pH 8.0. After heating (5 min, 95°C), 4 μl of the resolved protein were applied to sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) on a PhastSystem (Pharmacia Biotech), using 20% homogeneous polyacrylamide gels (PhastGel, Pharmacia Biotech). Focusing was carried out for 170 Vh at 15°C. Following transfer to nitrocellulose 0.2 μm membranes (Schleicher and Shuell, Düren), immunodetection of βTP was performed using a monoclonal mouse antibody against βTP (Prof. Conradt). The different fractions were detected by chemiluminescence with a horseradish peroxidase-labelled second goat anti-mouse antibody (Pierce, Hyperfilm-ECL).

2.2.5. Enzymatic release of N-glycans by peptide-N-glycosidase F (PNGase F)

Five μg of purified human βTP was incubated with 0.2 U of peptide-*N*-glycosidase F (PNGase F, Boehringer Mannheim, Germany) at 37°C [14]. The enzymatic reaction was stopped after 30 min, 60 min, 2 h, 4 h and 8 h by freezing at −20°C. Separation and immunoblotting of the protein was performed as described.

Stepwise cleavage of the *N*-glycan linkage of glycoproteins in purified human β-TP with peptide-*N*-glycosidase F (PNGase F) revealed the formation of both a- and monoglycosylated forms of βTP with increasing incubation times (data not shown). This illustrates that hypoglycosylated isoforms could be identified reliably by the method employed. The reliability of the method was further confirmed by running variable amounts (stepwise increase from 20 to 120 μl) of CSF from a patient with CDGS type Ia. The abnormal presence of both hypoglycosylated β-trace isoforms could be shown in all samples. Quantifying the bands by laser densitometry showed a stepwise increase of all bands with increasing amounts of CSF applied (data not shown). The response was not fully linear with the amount of protein applied.

3. Results

3.1. Glycosylation of β-trace protein in normal CSF

The βTP of CSF samples from control individuals migrated on SDS–PAGE as a single band of about 27 kDa representing the diglycosylated isoform (Fig. 1). The a- and monoglycosylated isoforms of βTP were not present or below 1% of the amount of the diglycosylated isoform.

3.2. Glycosylation of β-trace protein in CDGS type I patients

As shown in Fig. 1, βTP in CSF of all CDGS type I patients showed the di- (27 kDa) and mono- (24 kDa) glycosylated isoforms of βTP.

In all CDGS type Ia patients an additional band was present, representing the aglycosylated (21 kDa) isoform. The estimated ratio for the di-, mono- and aglycosylated fraction was 60–65%:30–35%:5–10%. This aglycosylated band could not be detected in any of the CDGS type Ic and Ix patients. In these cases the relative distribution of the di- and monoglycosylated isoforms was 70–85%:15–30%.

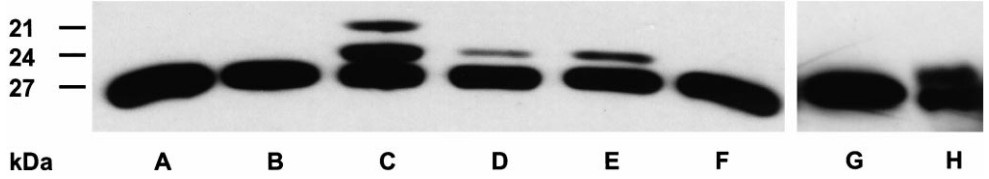

Fig. 1. Western blot analysis of β-trace protein in cerebrospinal fluid from CDGS patients of different subtypes: A, purified human βTP; B, control; C, CDG Ia; D, CDG Ic; E, CDG Ix; F, case H.K.; G, control; H, case C.V. Molecular mass of the isoforms is indicated in kDa.

3.3. Glycosylation of βTP in subject H.K.

In CSF of the mentally impaired patient H.K. with clearly abnormal glycosylation of several serum glycoproteins (for transferrin fractions see Table 1), a normal βTP pattern was observed with only diglycosylated βTP (Fig. 1).

3.4. Glycosylation of β-trace protein in subjects with neurologic disorders

Investigating the glycosylation of βTP in CSF from 500 children with unexplained disorders of the CNS, a 7-month-old child (C.V.) was identified with an abnormal pattern. In this patient the diglycosylated (27 kDa) isoform of βTP was reduced whereas the monoglycosylated (24 kDa) isoform was increased. The relative distribution amounted to 60:40 (Fig. 1) and was different from the distribution in patients with CDGS type Ia, Ic and Ix. Careful reinvestigations on serum glycoproteins (transferrin, TBG) revealed only marginal abnormalities as compared to normal controls (see Table 1). The clinical phenotype presented as follows. She was a dysmature newborn with neonatal seizures. Other major symptoms were severe muscular hypotonia, severe feeding problems, failure to thrive and hypoglycaemic episodes. Detailed differential diagnostic procedures, including comprehensive screening for metabolic disorders, did not lead to a diagnosis. Since the child died at 8 months of age before this βTP observation was carried out, no further investigations could be performed. Post-mortem examination revealed liver fibrosis with transition to cirrhosis and spleen infarctions. Examinations of the CNS showed no structural abnormalities, and routine light microscopic examinations were also unremarkable.

4. Discussion

The carbohydrate-deficient glycoprotein syndromes present as genetic multisystemic disorders characterised by defective glycosylation of the *N*-linked glycan units of a variety of glycoproteins. The most frequent CDGS type Ia (PMM deficiency) primarily affects the CNS and to a variable degree also the peripheral nervous system and other organs, e.g., liver. Neuropathology findings characteristically include cerebellar hypoplasia already in newborns and it is assumed that the regularly occurring arrest of locomotor ability in early childhood is related to a postnatal degeneration of infratentorial structures [24]. Neurological involvement appears less severe in CDGS type Ic and Ix patients. Abnormal βTP isoforms in CSF showed disturbed glycosylation in the CNS in CDGS type Ia but also in CDGS type Ic and Ix cases. We conclude that protein *N*-glycosylation is disturbed in the central nervous system and in peripheral organs in all CDGS type I subtypes investigated. Our data on CDGS Ia cases are in accordance with Pohl et al. [25] who demonstrated hypoglycosylation of βTP in CSF in CDGS type Ia and CDGS type II (*N*-acetylglucosaminyl-transferase II deficiency). Our study extends these findings to the CDGS Ic and Ix subtypes.

On the basis of the glycosylation pattern of βTP, we could distinguish between CDGS type Ia and other CDGS type I syndromes. The fact that among all CDGS type I syndromes the aglycosylated (21 kDa) isoform of βTP is only present in CSF of patients with CDGS type Ia may indicate that brain-type glycosylation is more severely impaired in these cases than in CDGS type Ic or Ix. The biochemical defect in the brain apparently is more deleterious in CDGS type Ia than in CDGS Ic and Ix. This is in line with the clinical observation of more severe neurological disturbances in patients with CDGS type Ia. In the CDGS type Ia cases there seems to be less residual flux through the 'brain-type' glycosylation pathway than in the CDGS type Ic and Ix cases. This may be explained by a higher local residual activity of the enzyme(s) involved in the CDGS type Ic and Ix cases. Of course it cannot be excluded that patients with more severe mutations in the gene underlying the CDGS type Ic or Ix defect may be found that will turn out to have a similar degree of impairment of the pathway as our CDGS type Ia cases.

As a result of investigations of *N*-glycosylation defects in brain of patients with impairment of the CNS of unknown aetiology, we identified one subject (H.K.) with an abnormal glycosylation pattern of different serum glycoproteins but with normal *N*-glycosylation of βTP in CSF. On the other hand, we found one subject (C.V.) with only very mild abnor-

malities of serum glycoproteins and a highly abnormal βTP glycosylation pattern. Known causes for secondarily impaired glycosylation of serum glycoproteins such as alcohol abuse, liver disease, hereditary fructose intolerance and galactosaemia were excluded [26–28]. In patient C.V. one may assume a glycosylation defect mainly confined to the CNS which to our knowledge has not been described so far. These patients demonstrate that abnormal *N*-glycosylation of proteins can occur restricted to either the 'peripheral' tissues or the brain.

Although the physiological role of βTP in CSF and its complete biosynthetic pathway still remain obscure, different studies on the biological function, especially in brain, indicate that it may play a critical role in regulating the development of neurones and may participate in myelin metabolism and maintenance [29]. βTP has been discussed as a diagnostic tool in various disorders. It is a specific marker for CSF fistulae and abnormal concentrations in CSF have been reported in cerebral infarction, multiple sclerosis, schizophrenia, paraproteinaemia and malignancies [30–34]. Whereas previous studies on βTP so far refer only to quantitative aspects, we here propose to extend its diagnostic relevance by studying βTP isoforms to light up impaired *N*-glycosylation of proteins in the CNS. Several defects in glycan synthesis have been described recently, and the number of newly discovered defects will rise as attention to these disorders increases and new methods are used. The method for the detection of abnormally glycosylated βTP in CSF is an easy and reliable test and can be used in a screening setting. It is well documented that isoelectric focusing of serum transferrin is the ideal approach to screen for CDGS [35]. βTP detection in CSF will not replace this approach, but this study suggests its diagnostic value for the screening for *N*-glycosylation defects in the CNS. As we have shown, these may even be exclusively brain tissue specific and would therefore be missed in the serum transferrin IEF analysis. Furthermore, the degree of βTP hypoglycosylation seems to reflect the severity of the CNS involvement.

βTP isoform analysis in CSF should be considered in the diagnostic workup of patients with an unexplained disorder of the CNS.

Acknowledgements

We acknowledge the collaboration of the patients and their families and of the referring clinicians. We are indebted to G. Matthijs and E. Schollen for the PMM mutation analysis. These investigations have been supported by a grant 'Stipendium Metabolicum' from the Milupa GmbH and Co., KG, Germany to Dr. S. Grünewald.

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